

Influence of the Dietary Level of Iron from Iron Methionine and Iron Sulfate on Immune Response and Resistance of Channel Catfish to *Edwardsiella ictaluri*

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Abstract

Channel catfish *Ictalurus punctatus* fingerlings were fed purified diets supplemented with iron at levels of 0, 20, 60, and 180 mg/kg from iron sulfate (FeS) or 5, 10, 20, 60, and 180 mg/kg from iron methionine (FeM) in triplicate tanks for 8 wk. Fish were then divided into two groups and subjected to different assays to measure disease resistance and individual immune functions. Representative fish from each dietary treatment were challenged by bacterial immersion with virulent *Edwardsiella ictaluri*, and mortality due to enteric septicemia was recorded. Other fish were immunized with 0.2-mL formalin-killed *E. ictaluri* and boosted 21 d post-immunization. Antibody response was determined by FAST-ELISA. Chemiluminescent and chemotaxis assays were performed using peritoneal macrophages. Supplementation of the diet with various levels of iron from FeS or FeM did not significantly affect antibody production. Chemotactic migration by macrophages was depressed in iron-deficient fish and a level of 60 mg/kg from either FeS or FeM provided the highest chemotactic indexes. A deficiency of dietary iron was found to increase mortality of channel catfish due to enteric septicemia of catfish (ESC). However, more studies should be conducted to better understand the effects of sources and levels of dietary iron on immune responses and disease resistance in channel catfish.

Culture of channel catfish *Ictalurus punctatus* resulted in gross farm revenues of 326 million dollars in 1993 (Aquaculture Situation and Outlook 1994). An additional 20–30 million dollars is estimated to be lost annually due to disease problems caused by *Edwardsiella ictaluri* (Plumb and Vinitnantharat 1993). *Edwardsiella ictaluri* was described by Hawke (1979) as the causative agent of the disease, enteric septicemia of catfish (ESC). Current methods of decreasing loss due to bacterial pathogens are limited to costly vaccines and antibacterial compounds administered through medicated feed. A more effective and economical method of control is needed to reduce losses.

The National Research Council (NRC) summarized the known minimum dietary requirements for most essential nutrients for maximum performance of channel catfish

(NRC 1993). However, studies have shown that increasing levels of some nutrients above minimum requirements improved immune responses and disease resistance in channel catfish. Durve and Lovell (1982) showed that increased levels of vitamin C reduced mortalities of channel catfish infected with *E. tarda*. Wise et al. (1993) showed that levels of vitamin E above the established minimum requirement improved phagocytosis of *E. ictaluri*. An improved understanding of the relationship between level and source of iron, and the immune response of channel catfish against *E. ictaluri* could provide a more effective method of decreasing losses due to ESC.

The minimum dietary iron requirement for optimum growth, feed efficiency, and blood values in non-diseased channel catfish has been determined to be 30-mg Fe/kg in purified diets (NRC 1993). Iron deficiency in channel catfish has been characterized by suppressed hematocrit, hemoglobin,

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plasma iron content and transferrin saturation (Gatlin and Wilson 1986). The effect of iron deficiency or excess on immune function in channel catfish has not been investigated.

The mechanism by which iron deficiency impairs immune responses in mammalian species are not clearly understood (Sherman 1984). Iron is crucial in mitosis and cell proliferation (Robbins et al. 1972). Iron deficiency is responsible for the reduced activity of several enzymes, including ribonucleotide reductase (Hoffbrand et al. 1976) and myeloperoxidase (Baggs and Miller 1973). Decreased protein synthesis due to the reduced activity of these enzymes may be a factor in reduced immunocompetence through decreased antibody production and decreased killing of bacteria by neutrophils (Sherman and Helyar 1988). Nalder et al. (1972) found a decreased antibody response to tetanus toxoid in iron-deficient rats. Moore and Humbert (1984) demonstrated subnormal phagocytic function in iron-deficient rats due to decreased bacterial killing and superoxide production.

Chelation of minerals to amino acids has been shown to increase the rate of absorption in animal intestines (Ashmead 1992). The efficacy of iron sulfate and iron methionine on immune function in channel catfish has not been examined.

The objectives of this study were: 1) to determine if dietary iron level from iron sulfate or iron methionine affects the antibody response and resistance of channel catfish to *Edwardsiella ictaluri*; and 2) to determine if the level and type of dietary iron affects functions of macrophages such as chemotaxis, phagocytosis, and bactericidal activity.

Materials and Methods

Experimental Animals

Channel catfish fingerlings (average weight, 8.5 g) from a single spawn, previously determined to be *E. ictaluri* free by culture (Klesius 1992) and serology (Kle-

sus 1993), were stocked in 110-L aquaria at a density of 60 fish per aquarium. The aquaria were supplied with flow-through well water at a rate of 0.6–0.8 L/min. Water temperature was maintained at 25–27°C with individual 300-watt aquarium heaters. Water quality and waterborne mineral levels during feeding period and immunological studies were found to be: pH, 7.6; nitrite, 0.4 mg/L; sodium, 21.9 mg/L; silicon, 13.4 mg/L; calcium, 18.6 mg/L; potassium, 1.9 mg/L; magnesium, 1.3 mg/L; and less than 0.5 mg/L for iron, manganese, zinc, boron, molybdenum, aluminum, barium, cobalt, chromium, copper, and phosphorus. Water was continuously aerated with an air blower through air stones. Photoperiod was maintained at 12:12 h light:dark schedule.

Experimental Diets and Design

Fish in randomly assigned triplicate tanks were fed egg-white-based basal diets modified from Gatlin and Wilson (1986) and supplemented with either 0, 5, 10, 20, 60, or 180-mg iron/kg of diet from iron methionine (FeM) (Zinpro, Chaska, Michigan, USA) or 20, 60, or 180-mg iron/kg of diet from iron sulfate (FeS) (Sigma Biochemical Co., St. Louis, Missouri, USA). Fish were fed experimental diets twice daily to apparent satiation for 2-wk pretrial, then for an additional 8 wk. Thereafter, fish continued to be fed experimental diets twice daily to apparent satiation. Iron content of the basal diet was determined to be 9.2-mg iron/kg diet using an inductively-coupled argon plasma (ICAP) spectrometer according to the method of Campbell and Plank (1992).

Bacterial Challenge

At the conclusion of the 8-wk feeding period, 25 fish from each of the original 110-L tanks (average weight, 35 g) were transferred into randomly assigned 57-L aquaria and allowed to acclimate for 5 d. Fish were challenged by immersion in a virulent isolate of *E. ictaluri* (AL-93-75) (Klesius and Sealey 1995). Mortality was recorded daily for 2 wk. Dead fish were col-

lected twice daily and assayed for presence of *E. ictaluri* (Klesius 1992). At 15 d post-challenge, remaining fish were euthanized.

Antibody Response

Channel catfish ($N = 35$, average weight = 35 g) remaining in each of the original 132-L aquaria were immunized intraperitoneally and boosted at 21 d with 0.2-mL formalin-killed *E. ictaluri* (1×10^6 /fish). Five fish were randomly selected from each tank, and bled prior to immunization and once weekly thereafter for 5 wk following anesthetizing with MS-222. Approximately 100- μ L serum/sample was collected following centrifugation at $1000 \times g$ for 5 min. The FAST-ELISA test (Klesius 1993) was employed to monitor the specific antibody response to *E. ictaluri*.

Peritoneal Exudate Cell Collection

Three fish from each of the original tanks were randomly selected, combined according to dietary treatment to give a total of $N = 9$ fish and transferred into 57-L aquaria. Fish were allowed to acclimate for 24 h then injected intraperitoneally with 0.25 mL of squalene (Sigma Chemical Co., St. Louis, Missouri, USA) per fish to elicit macrophages to the peritoneal cavity. Fish were returned to the aquaria for 5 d, then removed and anesthetized with MS-222 for cell collection. Cells were obtained by peritoneal wash with 10–15 mL sterile, chilled PBS. The supernatant was removed following centrifugation at $300 \times g$ for 10 min. The resultant pellet was then resuspended in 1 mL of 0.95% Hank's Balanced Salt Solution (without phenol red) (Gibco, Grand Island, New York, USA) for chemotaxis and chemiluminescence assays (Scott and Klesius 1981). Cell counts and viability were established following enumeration with a hemocytometer in 5% Trypan blue counting solution.

Chemiluminescence and Chemotaxis Assays

Chemiluminescence tests were performed by modifying the method of Scott

and Klesius (1981). Luminol-dependent chemiluminescence was performed in duplicate with peritoneal macrophages in a Pico-lite luminometer (Packard Instrument Co., Downers Grove, Illinois, USA). The control mixture contained $1.6\text{--}2.0 \times 10^5$ cells in 140- μ L 0.95% HBSS and 50 μ L of luminol. For zymosan and *E. ictaluri* test mixtures, the volume of HBSS was reduced and 50- μ L zymosan or 5- μ L *E. ictaluri* were added to maintain a final volume of 200 μ L. Phagocytic indexes were determined by dividing light emitted when zymosan and *E. ictaluri* were engulfed by light emission readings of control cells.

Chemotaxis assays were performed by modifying the method of Johnson et al. (1990). Assays were performed in quadruplicate using blind well chambers and 8- μ m polycarbonate membrane filters (Nucleopore, Pleasanton, California, USA) presoaked for 5 min in RPMI + 1% horse serum. In the bottom of test chambers 20- μ L *E. ictaluri* exoantigen (Klesius 1993) was added then supplemented with 180- μ L RPMI + 1% horse serum. In the bottom of control chambers only RPMI + 1% horse serum was added. Peritoneal macrophages were added to the top chamber at a concentration of $4.5\text{--}5.0 \times 10^5$ cells/chamber. Chambers were then incubated on a horizontal platform shaker for 90 min at 27 C. Following incubation, filters were removed, inverted and mounted on precleaned microscope slides. Slides were stained with Leukostat (Fisher Chemical, Pittsburgh, Pennsylvania, USA). Migrated macrophages were determined by counting five fields of duplicate filters at $1000 \times$ magnification.

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by Duncan's multiple-range test (SAS Institute Inc., 1993) to determine the differences between treatment means. Data were considered significant at the 0.05 probability level.

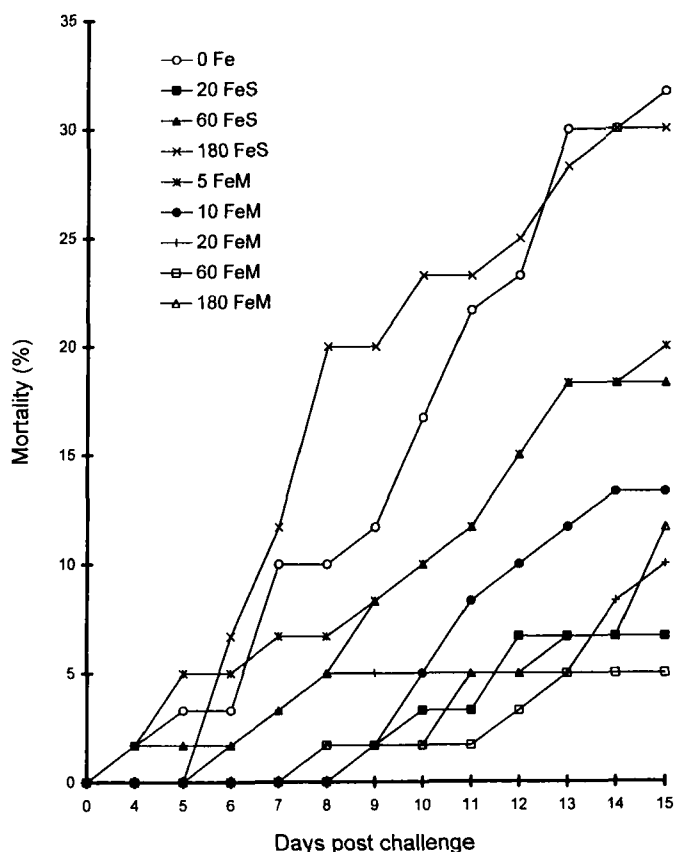


FIGURE 1. Mean daily cumulative mortality of channel catfish fed diets containing different levels of supplemental iron from iron methionine (FeM) or iron sulfate (FeS) following bacterial immersion with *E. ictaluri*.

Results

At the end of 8 wk, the average final weight of fish in different treatments ranged from 25.1 g to 32.2 g. Fish fed the basal diet without iron supplementation had significantly lower weight gain than fish fed diets containing supplemental iron from either FeM or FeS. Fish fed iron-supplemented diets did not differ with regard to final weight gain regardless of iron level. Fish fed the basal diet had significantly decreased hematocrit, hemoglobin, mean corpuscular hemoglobin and mean corpuscular volume. These values were also consistently low for fish fed diets with 5 and 10-mg iron/kg from iron methionine, but the differences were not always significant (Lim et al. 1996).

Bacterial Challenge

Fish fed the basal diet (0 Fe) and 5-FeM diet showed onset of mortality 3 d post-challenge which continued steadily throughout the 14-d monitoring period (Fig. 1). Fish fed the basal diet had the highest percentage total mortality (31.7%) which was significantly higher than that of fish fed the 60-FeM diet which had the lowest mortality (5.0%). These values, however, were not significantly different from those of fish fed the other diets.

Antibody Response

Prior to feeding trials, antibody titers were determined on sera from representative fish and no detectable antibody to *E. ictaluri* was observed.

TABLE 1. Mean antibody titer (OD) of immunized channel catfish fed diets containing different levels of supplemental iron from iron methionine (FeM) or iron sulfate (FeS). Values represent means of 4 fish/tank or 15 fish/treatment. Means with different superscripts in the same column are significantly different at $P < 0.05$.

Supplemental iron level (mg/kg diet)	Mean antibody titer (\pm SEM)	
	14 d post-immunization	14 d post-booster
0	0.20 \pm 0.50 ^a	0.29 \pm 0.03 ^{ab}
20 FeS	0.16 \pm 0.04 ^a	0.28 \pm 0.02 ^{ab}
60 FeS	0.25 \pm 0.06 ^a	0.30 \pm 0.02 ^{ab}
180 FeS	0.18 \pm 0.03 ^a	0.30 \pm 0.03 ^{ab}
5 FeM	0.26 \pm 0.03 ^a	0.23 \pm 0.03 ^b
10 FeM	0.22 \pm 0.04 ^a	0.28 \pm 0.02 ^{ab}
20 FeM	0.29 \pm 0.02 ^a	0.33 \pm 0.04 ^a
60 FeM	0.18 \pm 0.03 ^a	0.32 \pm 0.04 ^{ab}
180 FeM	0.17 \pm 0.03 ^a	0.31 \pm 0.03 ^{ab}

Specific humoral antibody titers of pre-immunized fish were below detectable levels. Antibody titers in response to formalin-killed *E. ictaluri* are shown in Table 1. No

significant differences in antibody responses occurred at 14 d post-immunization among treatment groups. At 14 d post-booster, fish fed diet supplemented with 20-mg iron/kg from FeM had significantly higher antibody titers than that of fish fed diet supplemented with 5-mg iron/kg from FeM. These values, however, were not significantly different from those of fish receiving other dietary treatments.

Kinetics of the immune response are shown in Fig. 2. Only data from fish fed the 0-Fe, 60-FeM and 180-FeS diets are presented and compared over the 5-wk monitoring period. Antibody levels following initial immunization peaked at week 2 and again at week 5 following booster immunization at week 3.

Chemiluminescence and Chemotaxis

Mean chemiluminescent indexes are presented in Table 2. Engulfment of zymosan was not significantly different among di-

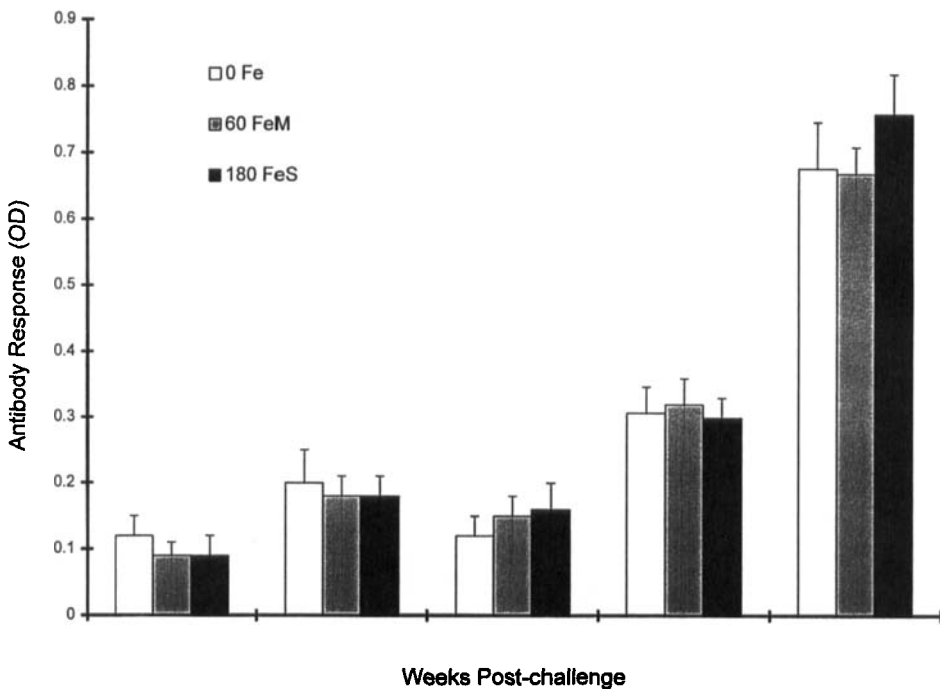


FIGURE 2. Kinetics of antibody responses of channel catfish fed diets containing no iron, 60-mg iron/kg from iron methionine (FeM), and 180-mg iron/kg from iron sulfate (FeS) following immunization with formalin-killed *E. ictaluri*.

TABLE 2. Mean chemiluminescent indexes for engulfment of zymosan and opsonized *E. ictaluri* by macrophages from channel catfish fed diets containing different levels of iron from iron methionine (FeM) or iron sulfate (FeS). Values represent means of duplicate samples. Means with different superscripts in the same column are significantly different at $P < 0.05$.

Supplemental iron level (mg/kg diet)	Chemiluminescent index (\pm SEM) ¹	
	Zymosan	<i>E. ictaluri</i>
0 Fe	1.99 \pm 0.05 ^a	4.25 \pm 0.00 ^{abc}
20 FeS	3.23 \pm 0.58 ^a	1.63 \pm 0.40 ^c
60 FeS	6.00 \pm 2.53 ^a	5.87 \pm 0.09 ^{ab}
180 FeS	2.97 \pm 1.47 ^a	4.14 \pm 1.34 ^{abc}
5 FeM	7.38 \pm 1.19 ^a	3.60 \pm 0.64 ^{abc}
10 FeM	2.10 \pm 0.33 ^a	2.33 \pm 0.82 ^{bc}
20 FeM	1.08 \pm 0.02 ^a	2.92 \pm 1.88 ^{abc}
60 FeM	3.99 \pm 1.68 ^a	6.66 \pm 0.00 ^a
180 FeM	7.36 \pm 4.72 ^a	2.08 \pm 0.04 ^{bc}

¹ Chemiluminescent indexes were determined by dividing counts/min when zymosan and *E. ictaluri* were engulfed by counts/min readings when no stimulant was present.

TABLE 3. Mean macrophage migration and chemotaxis index of channel catfish fed diets containing different levels of supplemental iron from iron methionine (FeM) and iron sulfate (FeS).

Supplemental iron level (mg/kg diet)	Mean macrophage migration (\pm SEM) ¹		Macrophage chemotaxis index ²
	0- μ g exoantigen	20- μ g exoantigen	
0 Fe	10.4 ^{ab}	10.4 \pm 0.6 ^e	1.0
20 FeS	13.0 \pm 4.8 ^{ab}	45.6 \pm 4.8 ^a	3.5
60 FeS	7.1 \pm 1.3 ^b	29.4 ^{bcd}	4.1
180 FeS	16.5 \pm 1.0 ^a	43.7 \pm 6.8 ^{ab}	2.6
5 FeM	10.9 \pm 0.3 ^{ab}	14.6 \pm 0.8 ^{de}	1.3
10 FeM	8.4 ^{ab}	17.8 \pm 1.4 ^{de}	2.1
20 FeM	13.3 \pm 0.1 ^{ab}	36.5 \pm 3.3 ^{abc}	2.7
60 FeM	9.0 \pm 0.4 ^{ab}	27.3 \pm 2.0 ^{cd}	3.0
180 FeM	17.2 \pm 3.0 ^a	34.9 \pm 1.4 ^{abc}	2.0

¹ Values represent means of 1 to 4 determinations/treatment. Means with different superscripts in the same column are significantly different at $P < 0.05$.

² Macrophage chemotaxis index was determined by dividing mean number of macrophages migrating in the presence of exoantigen by mean number of macrophages migrating in the absence exoantigen.

etary treatments. Peritoneal macrophages harvested from fish fed the 60-FeM diet exhibited a greater ability to engulf opsonized *E. ictaluri* than those of fish receiving other dietary treatments. Engulfment of opsonized *E. ictaluri* by macrophages from fish fed the 60-FeM diet was significantly higher than those of macrophages from fish fed the 20-FeS, 10-FeM and 180-FeM diets. The value for fish fed the 60-FeS diet was also significantly higher than that of fish fed the 20-FeS diet.

Macrophages from fish fed diets supplemented with 180-mg iron/kg from both FeM and FeS showed increased chemokinesis as shown by higher levels of migrating macrophages (17.2 and 16.5 macrophages/field, respectively) when no exoantigen was added (Table 3). In the presence of exoantigen, chemotaxis was lowest (10.4 macrophages/field; chemotactic index = 1) in macrophages from fish receiving no supplemental iron. Macrophage migration toward the exoantigen, as shown by the chemotactic index, increased with increasing levels of iron supplementation up to 60

mg/kg diet from either FeS or FeM. Index values decreased when either source of dietary iron level was increased to 180 mg/kg.

Discussion

Dietary-induced iron deficiency in channel catfish resulted in increased mortality when fish were challenged by bath immersion with *E. ictaluri*. Fish fed the 0-Fe (basal) diet were more susceptible to *E. ictaluri*. These results agree with those reported by MacKay (1928) who found an increased incidence of respiratory infections in iron-deficient infants. However, supplementation of the basal diet with 180-mg iron/kg from FeS resulted in increased mortality. Weinberg (1992) showed that iron-overloaded hosts are at an elevated risk to intracellular microbial infection. Studies by Shotts et al. (1986) and Miyasaki and Plumb (1985) indicated that *E. ictaluri* can live inside channel catfish phagocytes and facilitate disease by helping disseminate the organism via circulation (Klesius 1992). Whether supple-

mentation of 180-mg iron from FeS suppressed the immune response of the experimentally challenged fish or enhanced invasion and growth of the bacteria is unclear. It is equally difficult to explain why this phenomenon did not occur in fish fed the 180-FeM diet. This may be due to a decreased ability of *E. ictaluri* to breakdown and absorb chelated iron.

Iron deficiency did not significantly suppress specific antibody levels mounted in response to formalin-killed *E. ictaluri* measured by FAST-ELISA. Similar results were observed by Chandra (1975) who demonstrated normal or elevated concentrations of IgG, IgA, and IgM in iron-deficient humans. She also showed unaffected levels of specific antibodies to tetanus toxoid, diphtheria, and *Salmonella typhi*. These results contrast to those of Nalder et al. (1972) who found iron deficiency significantly reduced the specific antibody response of rats to tetanus toxoid. The relatively low antibody level in fish fed the diet supplemented with 5-mg iron/kg from FeM at 14 d post booster could not be explained since the corresponding value at 14 d post-immunization was not low. These results suggest that specific antibody titer was not affected by either the source or the level of dietary iron. Kinetics of the antibody response of fish fed 60 FeM and 180 FeS are similar to those observed by Klesius and Sealey (1995) and indicate the kinetics of the antibody response were largely unaffected by type and level of dietary iron.

Engulfment of bacteria by macrophages as measured by chemiluminescence was not significantly depressed by iron deficiency. It appeared, however, that maximum phagocytic engulfment of opsonized *E. ictaluri* by macrophages was obtained from fish fed 60 mg supplemental iron/kg from either FeM or FeS, suggesting that the source of dietary iron had no significant effect on engulfment.

Macrophages from fish receiving the basal diet with no iron supplementation had significantly lower migration (in the pres-

ence of exoantigen) than those from fish fed all other diets with the exception of the 5-FeM diet which was also mildly iron deficient based on hematological values as has been reported by Lim et al. (1996). Iron supplementation at a level of 180-mg iron/kg from both FeS or FeM also increased chemokinetic activity of macrophages implying that iron level does play some role in macrophage migration. This role can be further demonstrated by the chemotactic index which shows a trend in which migration increases to a peak and then decreases possibly indicating suppression of the response.

Results of this study indicate that, in interactions between channel catfish and *E. ictaluri*, iron source and level influences the physiological status of the fish. The total dietary iron of 30 mg/kg diet required for maximum growth of channel catfish reported by the National Research Council (1993) may be sufficient for an optimum immune response. However, an optimum level and source of dietary iron for maximizing the immune response is complicated by the different optimum dietary levels for individual immune parameters. Iron deficiency increased susceptibility of channel catfish to *E. ictaluri* infection, but increasing dietary iron levels above the requirement of 30 mg/kg was not protective. In addition, higher levels of iron supplementation (180 FeS) may also lead to increased susceptibility.

Commercial catfish production diets contain approximately 118-mg iron/kg diet (Gatlin and Wilson 1986). Depending on bioavailability of iron in commercial feeds, actual levels may be at or above levels required for an optimum immune response against *E. ictaluri*. Therefore, supplementation of iron in commercial diets of channel catfish may lead to iron-overload, which as shown in this study, suppresses some immune functions and may increase mortality. Further studies are needed to elucidate the bioavailability and optimum level of iron in commercial diets of channel catfish and its

effect on immune response and disease resistance.

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